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Stickleback brain size and behaviour in relation to the physicochemical properties of water

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Abstract

It is becoming ever more recognised that global climate change is intensifying the symptoms of eutrophication in freshwaters causing changes in the visual and chemical properties of marine habitats. It is expected that human-induced environmental change will act to degrade the sensory environment by masking signals and their reception. However, this environmental change could have the potential result in adaptation towards novel communication traits or change the traits that are under selection. It was suggested that brains are plastic structures that are manipulated by experience and this neurological change will act to alter the behaviour of the individual. Brain plasticity has since been demonstrated in a number of studies but so far the links between the two have merely been suggestive and needs to be studied unequivocally. Additionally, sensory preference in numerical cognition has never been explored. This study aimed to bridge this gap. The first experiment is one that examines how brain size varies between populations of the same species. Three-spined stickleback specimens were taken from 10 lochs of North Uist, selected for their differing characteristics. Their whole brains and various individual brain regions were then dissected and measured. It was shown that overall brain size was affected by social complexity and the olfactory region was affected by the pH of the water, with more alkaline lochs producing fish with larger olfactory bulbs. In the next experiment, 9-spined sticklebacks were house in two treatments, dark and clear water. The fish were given sensory preference trials through the use of a series of numerosity trials that gave them a choice between two shoals, where the ratio of individuals ranged from 0.4 to 0.8. The olfactory and visual stimuli were presented diametrically and simultaneously. The fish were then dissected and their brains measured. Fish housed in dark water had brains that were that were the same size as fish in clear water but had smaller optic tectum and larger olfactory bulbs than those in clear water. Neither dark nor clear water reared fish showed any deviation from chance and thus showed no preference for either vision or olfaction for shoaling at any ratio. However, when the data was examined further in terms of 1st choice, it was seen that the dark water fish showed an affinity towards the olfactory stimulus and the clear water group showed an affinity for the visual stimulus.

This indicates that the behaviour had in fact started to deviate in relation to the associated sensory brain region. On a broad spectrum, what we have shown here is that individuals possess the ability to adapt their behaviour and morphology to that what is most appropriate to the environment in which they find themselves. Climate change is affecting the planet as a whole but places are changing differently to others in terms of temperature, acidity and so on and this may give rise to speciation through the different ways that populations of the same species adapt their modalities of such behaviours as communication, shoaling and mate choice.

1.0: General Introduction

It is becoming ever more recognised that global climate change is intensifying the symptoms of eutrophication in freshwaters (Jeppesen *et al.,* 2010a; b; Moss, *et al.,* 2011). Eutrophication is a natural process whereby a body of water acquires a high concentration of nutrients, especially phosphates and nitrates (Art, 1993); however climate change, by intensifying storms, affecting rainfall patterns, warming soils and melting glaciers, will increase diffuse nutrient loading (Jeppesen *et al.,* 2011; Moss, *et al.,* 2011) causing algae blooms that cloud water columns and increase sediment suspension in the environment. Moreover, runoff from anthropogenic practices, including agriculture, silviculture, urbanization, mining, and road construction, greatly contribute to an increase in sediment loads and eutrophication (Waters, 1995; Sutherland, *et al.,* 2002; Roy, *et al.,* 2003), all of which act to increase turbidity and pH levels in aquatic environments (Mason, 2002). Turbidity is the most common measure of suspended sediment in streams, representing the amount of light scattered or absorbed by a water sample (Duchrow and Everhart, 1971). A reduction in the availability of light within an environment brought about by such factors as an increase in turbidity not only has many deleterious effects on fish health (See Henley, *et al.,* 2000) but also the associated reduction in the amount of light available and shifts in the colour of underwater light (Lythgoe, 1979; 1988; Seehausen and Schluter, 2003; Utne-Palm, 2002) act to limit fish vision by reducing the visual distance and perception, interfering with social behaviour (Berg and Northcote, 1985), foraging behaviour (Gregory and Northcote 1993; Vogel and Beauchamp, 1999) and predator avoidance (Miner and Stein, 1996; Meager, *et al.,* 2006). Zamor (2002) showed that increased degradation of the visual environment acts to reduce the reactive distance and prey capture success of rosyside dace (*Clinostomus funduloides*).

Another issue is the amount of CO_2 and other greenhouse gases being produced through anthropogenic practices. When carbon dioxide (CO_2) is absorbed by water, chemical reactions occur that reduce water pH, carbonate ion concentration, and saturation states of biologically important calcium carbonate minerals (Doney, *et al.,* 2009; Feely, *et al.,* 2009). Since the beginning of the

Industrial Revolution, the pH of surface ocean waters has fallen by 0.1 pH units. This change represents approximately a 30 percent increase in acidity. Future predictions indicate that the oceans will continue to absorb carbon dioxide and become even more acidic (Doney, *et al.,* 2009).

These factors not only affect the physical environment but also act to alter the sensory environment of organisms inhabiting marine habitats. Changes in the sensory environment influence animal communication by modifying how signals are sent (e.g., endocrine disrupting chemicals altering the expression of sexually selected ornamentation; Baatrup and Junge 2001), by altering signal transmission (e.g., noise pollution masking acoustic signals; Slabbekoorn and Peet 2003), or by affecting an animal's capacity to receive the signal due to shifts in receptor sensitivity (e.g. increased turbidity and changes to wavelength composition driving shifts in visual sensitivity; Hoffmann *et al.,* 2009). It is expected that human-induced environmental change will act to degrade the sensory environment by masking signals and their reception. However, this environmental change could have the potential result in adaptation towards novel communication traits or change the traits that are under selection. For example, as a result of eutrophication the increased pH enhances female preference for male odour cues over male visual displays in sticklebacks (Heuschele and Candolin, 2007). Sticklebacks have also been shown to switch from visual to chemical signalling methods in turbid environments (Heuschele, *et al.,* 2009). i.e. when in a visually restrictive environment, sticklebacks maintain the ability to communicate using chemical cues as the signal will be much more effective than a visual one.

Behaviour has been described as being a plastic phenotypic trait and is often the first mechanism used to deal with environmental stressors (West-Eberhard, 2003; Ghalambor *et al.,* 2010; Sih *et al.,* 2011), i.e. upon contact with an environmental stressor or novel environment, animals have the ability to adjust their behaviours accordingly to lessen the effects of the environmental change. Different kinds of experience during early life can play a significant role in the development of an animal's behaviour. In natural contexts, this influences behaviours from anti-predator responses to navigation abilities

(Salvanes, *et al.,* 2013). Alternatively, increased energy expenditure on activities related to reproduction may be necessary to offset difficulties in communication caused by the indirect or direct effects of an environmental stressor. For example, three-spine stickleback (*Gasterosteus aculeatus*) males in the Baltic Sea experiencing increased eutrophication turbidity spend more time courting than males in clear water, with no net gain in reproductive success (Candolin, *et al.,* 2007). In addition, turbidity apparently reduced the perceived risk of predation in juvenile Chinook salmon (*Oncorhynchus tshawytscha*) due to the reduction in visual distance (Gregory, 2003) and Fischer and Frommen (2013) found that 3-spined sticklebacks in turbid water showed no preference for shoal size whereas in clear water they showed a significant preference for larger shoals. Brown, *et al.,* (2002) found that the ability of fathead minnows (*Pimephales promelas*) and finescale dace *(Phoxinus neogaeus*) to detect and respond to conspecific and artificial alarm pheromones was lost when the pH of their environment was buffered to 6.0. This suggests that more acidic environments act to vitiate the chemical signal either directly on the transmitter and/or the receiver or is degraded within the environment.

Differences in behaviour and cognitive abilities are often attributed to differences in the morphology of the associated brain region. For example, numerous studies have linked the relative size of the telencephalon to spatial learning and cognitive mapping (see Salas *et al.,* 2003). The ecological demand for increased spatial learning has shown to affect telencephalon size in cichlids where larger sizes were seen in the species that resided in more complex habitats consisting of shallow rock and vegetation than those found in simpler environments (Huber, *et al.,* 1997; Van Staaden, *et al.,* 1994) Early studies of Neuroscience suggested that the brain is a physiologically static organ that is fixed after a critical period in development (Pascual-Leone, and Hamilton, 2005). This has since been largely discredited due to a large body of evidence in support of Neuroplasticity, which explores the ways in which the brain changes throughout life (Pascual-Leone and Hamilton, 2005). In a review by Kolb and Whishaw (1998) it was suggested that brains are plastic structures that are manipulated by experience and this neurological change will act to alter the behaviour of the individual. Brain plasticity has since

been demonstrated in a number of studies (Gonda, *et al.,* 2009; 2010a,b; 2011; 2013; Park and Bell, 2010; Pollen, *et al.,* 2007) but so far the links between brain and behavioural plasticity have merely been suggestive and needs to be studied unequivocally. Therefore the purpose of this study was to bridge the gap between brain morphology and behaviour (or more specifically, sensory preference) and how this can be manipulated by the environment. This allowed us to then make predictions as to how marine species will adapt to the changes caused by climate change.

Ecological correlates of brain size in 3-spined sticklebacks

2.1: Introduction

Brain Plasticity

Intraspecific variation in brain size has been revealed amongst a diverse range of taxonomic groups, including primates (Harvey, *et al.,* 1980), fish (Kotrschal, *et al.,* 1998), and birds (Day, *et al.,* 2005). Such studies have given insight into the relationships between brain morphologies and ecological factors and behavioural and life history characteristics associated with fitness (Gonda, *et al.,* 2009). Habitat (Pollen, *et al.,* 2007) and social (Gonzalez-Voyer, *et al.,* 2009) complexity are amongst the factors that have been found to be associated with brain size with interspecific comparisons forming the basis of what has already been studied in this field (see Gonda, *et al.,* 2012). Kihslinger *et al.,* (2006) and Lisney *et al.,* (2007) suggested that due to energetic constraints, those regions of the brain that are likely to most suited to the habitat or social environment will develop more than those that are of less importance. It is expected that we will see differences in relative brain size and individual region size dependent on the different ecological and social environments in which different populations of 3-spined sticklebacks exist

Energetic constraints, as a consequence of brain tissue being extremely energetically expensive to maintain (Aiello and Wheeler, 1995; see also Navaterre, *et al.,* 2011; Allen and Kay, 2012; Warren and Iglesias, 2012; Kotrschal, *et al.,* 2013), should inflict strong selective pressure against non-adaptive variations. This means that an increase in brain size can only ensue when the benefits of a larger brain compensate for the cost of production and maintenance (Safi, *et al.,* 2005). For example, selection for increased cognitive ability should favour increased brain size, but this can only occur if enough resources are available to meet the increased energetic demands without any loss to other aspects of fitness. For this reason, the size of any individual brain region could potentially be indicative of its relative importance, and reflect the way the given species or population has adapted to its

environment and prevailing selective regime (Krebs, *et al.,* 1989; de Winter and Oxnard, 2001; Gonzalez- Voyer and Kolm, 2010).

Studies on brain development have shown that those regions of the brain that are likely to be important in a particular environment develop more than those that are of less importance (Kihslinger and Nevitt, 2006; Kihslinger, *et al.,* 2006; Lisney, *et al.,* 2007). Differences in habitat, diet, or behaviour have been shown to alter the relative size of the main sensory brain areas in fish (Wagner, 2003; Lisney, *et al.,* 2007). It would seem that changes in demand predominantly alter the number and sizes of component elements rather than their connectivity (Kotrschal and Junger, 1988; Huber and Rylander, 1992), making the relative size of brain areas a reliable predictor of their relative importance (Kishida, 1979; Kotrschal and Palzenberger, 1992; Schellart, 1992; Schellart and Prins, 1993). For example, captive rearing has been shown to reduce total brain size in guppies, *Poecilia reticulata* (Burns and Rodd, 2008), size of the olfactory bulb; structure located in the forebrain of vertebrates that receives neural input about odours detected by cells in the nasal cavity; (Diaz, *et al.,* 2011; 2013) and telencephalon; the dorsal portion of the forebrain that mediates a variety of behaviours such as classical conditioning (Flood, *et al.,* 1976; Overmier and Hollis, 1983) and processing of special and sensory information (Davis and Kassel, 1983), in Chinook salmon, *Oncorhynchus tshawytscha* (Kihslinger, *et al.,* 2006) and guppies (Burns and Rodd, 2008). It has also been shown to reduce the relative size of every main brain part as well as the size of the whole brain in nine-spined sticklebacks, *Pungitius pungitius* (Gonda, *et al.,* 2011). Additionally, it was shown by Huber, *et al.,* (1997) that in African cichlids, the olfactory bulb correlated positively with water turbidity due to the degradation of the visual signal produced and received by conspecifics within the environment.

Correlations have been revealed between brain architecture and different biological influences across species (e.g., Pollen *et al.,* 2007) such as, seasonality (van Woerden, *et al.,* 2010), life history (Gonzalez-Voyer *et al.,* 2009a; Isler, 2011; Barton and Capellini, 2012), sexual selection (*Fitzpatrick et al.,* 2012),

behavioural plasticity (Ratcliffe *et al.,* 2006; Aviles and Garamszegi, 2007), and morphological traits (eg. positive correlations with body size, Gonzalez-Voyer *et al.,* 2009b; and negative correlations with gut size, Aiello and Wheeler 1995).

Many important biotic factors such as social environment, predation risk, or competition have rarely been explored (but see, e.g., Gonda, *et al.,* 2009a, 2010, 2012; Trokovic, *et al.,* 2011). It has been shown that social environment can alter brain development, especially the sensory brain areas, both in the nine-spined stickleback (Gonda, *et al.,* 2009a) and the common frog (*Rana temporaria*; Gonda *et al.,* 2010; Trokovic, *et al.,* 2011). For example, 9-spined sticklebacks reared independently in tanks that allowed water flow through, developed smaller optic tectum and larger olfactory bulbs than fish reared in a group due to lack of visual stimuli and the presence of olfactory stimuli from other compartments, and in some highly aggressive populations group rearing resulted in decreased overall brain size due to increased competition between individuals and the expensive energy requirements of the brain (Gonda *et al.,* 2009a). The development of the main sensory brain areas were also affected by group density in both tadpoles and metamorphosed froglets (Gonda *et al.,* 2010; Trokovic *et al.,* 2011), in that tadpoles grown at low density and under predation risk developed smaller brains than tadpoles at the other treatment combinations. Further, at high densities, tadpoles developed larger optical lobes and smaller dorsal medulla than those grown at low densities. The implications of this are that at lower densities, the tadpoles engage in less social interactions than if at higher densities causing the development of smaller brains, adhering to the social brain hypothesis (Section 2.1.2; Dunbar, 1998; Dunbar, 1993).

2.1.1: Study Species

In this study 3-spined sticklebacks *G. aculeatus* were used as the model system. The 3-spined stickleback is appropriate species for studies such as this because they are high in abundance throughout much of the northern hemisphere found in a range of different habitats from salt water

to freshwater (Gonda, *et al.,* 2011; Kingsley, 2003) and the neurogenesis persists throughout ontogenesis contributing the lifelong growth of brain size and the increased potential for plastic responses to environmental variation (Gonda, *et al.,* 2011). They are useful model systems for brain structure because the brain of the 3-spined stickleback, like in other fish species, is quite simple. It can be divided into 6 distinct regions which are visible with the naked eye (Pollen, *et al.,* 2007) each with its own distinct set of functions which can be related to its corresponding sensory system.

2.1.2: Objectives

The aim of this study was to investigate if brain size and the size of individual brain regions could be affected by the social and ecological factors of the environment. Based on the findings of such studies as Pollen *et al.,* (2007) and Gonda *et al.,* (2009a;b; 2011; 2013) that have shown differences in brain architecture depending on social and ecological factors and the suggestion by Kihslinger and Nevitt (2006), Kihslinger *et al.,* (2006) and Lisney et al., (2007) that due to energetic constraints, those regions of the brain that are likely to be important in a particular environment develop more than those that are of less importance, it is expected that we will see differences in relative brain size and individual region size dependent on the different ecological and social environments in which different populations of 3-spined sticklebacks exist

In particular, we would expect to see a habitat induced trade-off between vision and olfaction through the olfactory bulb and the optic tectum in wild caught 3-spined sticklebacks. Amongst the lochs of North Uist, Scotland there is a strong axis of variation in pH associated with the variation in concentration of alkaline metals (MacColl, *et al.,* 2012). Chemical cues are integral to many species of shoaling fish, which are strongly attracted towards the smell of conspecifics (Ranta, *et al.,* 1992; Ward *et al.,* 2007). Other studies suggest that chemical cues are of greater importance than visual cues, especially for longer range detection (Kleinhappel, *et al.,* 2014; Ward *et al.,* 2002). Chemical cues give individuals the ability to discriminate between conspecifics very specifically and are fundamental in maintaining their patterns of social structure, such as shoaling and dominance hierarchies (Todd *et al.,* 1967; Courtenay *et al.,* 1997; Wyatt 2003; Ward *et al.,* 2007). Heuschele and Candolin (2007),

Davison (2013) and Antoine, *et al.,* (2013) suggested that factors such as increased pH can boost olfactory signalling in sticklebacks and while low pH values have been shown to cause irreversible disruptions of fish cues, through alterations in the structure of the transmitting molecules (Brown, *et al.,* 2002; Leduc, *et, al.*, 2004). Munday, *et al.,* (2008) supported this when they showed larval clownfish (*Amphiprion ocellaris*) reared in pH 8.15 discriminated between a range of cues that could help them locate reef habitat and suitable settlement sites. This discriminatory ability was disrupted and larvae became strongly attracted to olfactory stimuli they normally avoided when reared at pH 7.8 and they no longer responded to any olfactory cues when reared at pH levels pH 7.6. Therefore, it was predicted that the relative size of the olfactory bulb would scale positively with increased pH. If the intensity or colour of available light is altered due to, for example, suspended particles that scatter the light, visual communication will be compromised (Ranaker, *et al,* 2012; van der Sluijs *et al.,* 2008a). Van der Sluijs *et al.,* (2008a) proposes that the value of a visual signal relies on the intensity and spectral composition (colour) of light upon the signaller, transmission through the medium, background light, and detection by the visual system of the receiver. If the intensity or colour of available light is altered due to, for example, suspended particles that scatter the light, visual communication will be compromised (van der Sluijs *et al.,* 2008a). It was shown in 3-spined sticklebacks that darker waters brought about by eutrophication, debilitated the strength of sexual selection based on visual traits, increased the cost of mating, and allowed dishonest visual communication of male condition (Candolin *et al.,* 2007; Wong *et al.,* 2007). However, fish visual systems have been shown to be phenotypically plastic (salmon: Cheng and Flamarique (2004); cichlids: Wagner and Kroger, 2005), suggesting a capacity for changing environmental condition to initiate immediate morphological and physiological changes. It was therefore predicted that the relative size of the optic tectum would correlate negatively with increased absorbance of the water sample from its environment.

Another variable that is suggested to act upon brain size is complexity of social interactions. The social brain hypothesis states that an individual's ability to manage complex relational information is dependent on its cognitive capacity and therefore, on brain size (Dunbar, 1998). It is known that 3- and 9-spined sticklebacks regularly shoal together, thus an increased number of 9-spines can be used as a proxy for social complexity in the environment as the increased proportion may cause a more complex series of social interactions requiring an increased cognitive capacity to deal with pressures such as species recognition, increased competition for food or even may be an indication of greater species diversity within the environment (Pollen, *et al.,* 2007; Reader and Laland, 2002).

2.1.3: Hypotheses

To address the aims of this study, the following hypotheses were tested: (1) the relative size of the olfactory bulb will scale positively with increased pH; (2) the relative size of the optic tectum will correlate negatively with increased absorbance of the water sample from its environment; and finally, (3) total brain volume would increase with increased social complexity, with the ratio of heterospecifics (9-spined sticklebacks) used as a proxy.

3.1: Method

3.1.1: Study Location

The location selected for taking fish samples was North Uist, Outer Hebrides, Scotland. The reason for this was that there are many lochs with a strong axis of variation in ecological characteristics including. These lochs are isolated yet are within close proximity to one another making sampling from them easier and more efficient (MacColl, *et al.,* 2012).

Figure 1: The Location of North Uist, Scotland.

3.1.2: Field Sampling

Two hundred specimens of 3-spined sticklebacks were collected by a fellow researcher from 10 lochs (20 from each) in North Uist, Scotland, using un-baited 1/4'' GEE wire mesh minnow traps set out for 48hours in October 2013 (Table 1). Lochs were selected based on the variation in ecological factors including water pH, absorbance, known abundance of 9-spined sticklebacks and whether or not populations of 3-spined sticklebacks are known to exist there. Locations and ecological information of pH, relative abundance of 9-spined sticklebacks for the samples came from MacColl *et al.,* (2012) with absorbance being measured by taking water samples back to the lab and using a light spectrometer calibrated to a wavelength of 450nm (see Table 1). There are limitations to this in that the project relies on the ability of another researcher to provide sound data but due to the fact that fluctuations do occur in these variables and MacColl *et al.,* (2012) took measurements over a series of months and provided the average, it seemed that the data provided in that study would be more reliable than one-off measurements. All specimens were euthanized on site immediately after capture with an overdose with MS-222. Immediately following euthanasia, Specimens from the 10 populations were fixed in

10% formalin in 0.1 mM (10.9 g dibasic: 2.5 g monobasic: 1 L deionized water) sodium phosphate buffer solution (PBS), which is isotonic with fish tissue and buffered at pH 7.4. This fixation protocol does not appear to affect neuro-morphology significantly according to Park and Bell (2010) and Gonda, *et.al.* (2009, 2011, 2012).

Loch	Eubal	Moracha	Hosta	Abuird	AnDaimh	Abharpa	Saandaraigh	Sgaraigh	Sgdabhagh	Feithean
Abs.	0.034	0.013	0.007	0.026	0.029	0.022	0.032	0.061	0.007	0.013

Table 1: Measured absorbance values for each loch.

Loch	Eubal	Moracha	Hosta	Abuird	AnDaimh	Abharpa	Saandaraigh	Sgaraigh	Sgdabhagh	Feithean
pH	8.5	6.3	8.3	6.1	6.5	6	8.3	8.5	6.1	8.3

Table 2: Measured pH values for each loch.

Loch	Eubal	Moracha	Hosta	Abuird	AnDaimh	Abharpa	Saandaraigh	Sgaraigh	Sgdabhagh	Feithean
%	24	0	25	5	17	0	7	0	11	53

Table 3: Relative *pugitus* % values for each loch.

3.1.3: Brain Measurements

All 200 specimens were sampled with one specimen chosen at random from each group for dissection at a time. Standard length, defined as the distance from the tip of the upper jaw to the end of the vertebral column, was measured to the nearest 0.01mm using ImageJ software after a photograph was taken of the specimen under an imaging microscope. Specimens were placed under a dissecting microscope, and their brains were extracted dorsally after removing the parietal, frontal and nasal bones (Park and Bell, 2010). Digital images of the dorsal and lateral aspects of the brain were taken using a computer-assisted video image analysis system, Motic Images 2.0. Images were always 800x600pixels, magnification 8 and were saved in JPeg format.

The length (L), width (W) and height (H) of all brain regions were measured to the nearest 0.01μm using ImageJ software (see below). Height and width were measured as the greatest distance perpendicular to the brain and length was measured as the greatest distance parallel to the brain. The volume (V) (μm^3) of each brain region and absolute brain size was calculated using the ellipsoid model (Pollen, 2007; Gonda *et al.,* 2009, 2010, 2011, 2012):

$$V= (L * W * H)/6.$$

Following that, The relative brain size was calculated by dividing the absolute brain size by the standard length which had been converted to μm. the relative size of each individual brain region was calculated by dividing the size of the brain region by the absolute brain size.

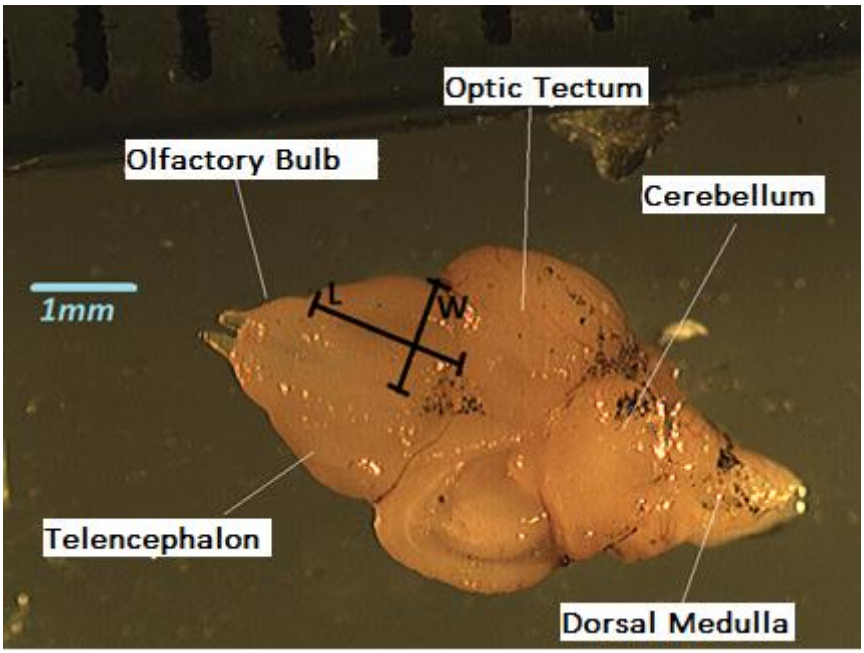

Figure 2: The stickleback brain and its individual regions.

3.1.4: Data Analysis

Firstly, all data was tested for normality using the Kolmogorov-Smirnov and the Shapiro-Wilk tests. Any data that was not normally distributed ($p>0.05$) was Log10 Transformed and retested. All data was successfully normalised using Log10. Then the relative brain size and each individual brain region

was analysed at the loch level using an ANOVA. This allowed for the identification of brain size and regional differences between lochs and a post hock tukey test highlighted where the differences were. Following this, a General Linear Model (GLM) was used with Relative Brain Size, olfactory bulb and optic tectum and as the dependent variables, Loch as the random factor, and Loch pH, Absorbance and Relative *Pungitus* Abundance as covariates. All analyses were carried out using SPSS ver. 19.

4.1: Results

4.1.1: Fish Length

There was no significant difference between the length of the fish measured in the samples from each loch ($F_{(1,136)}$=1.897 , p=0.12).

4.1.2: Brain Size

There were significant differences in relative brain sizes between lochs ($F_{(1,136)}=11.025,\ p<0.001$) (figure 3a). These differences in relative brain size were being driven by pH ($F_{(1,134)}=24.763,\ p<0.001$) (figure 3b), and relative *Pungitus* abundance ($F_{(1,134)}=18.81,\ p<0.001$) (figure 3c)

There were no significant differences between relative optic tectum ($F_{(9,124)}=1.638,\ p=0.112$), telencephalon (ANOVA: $F_{(9,124)}=0.679,\ p=0.726$) or cerebellum ($F_{(9,124)}=1.483,\ p=0.162$) sizes between lochs. There was a significant difference between relative olfactory bulb sizes ($F_{(9,124)}=8.633,\ p<0.001$) (figure 4a) between lochs and these differences were driven by loch pH ($F_{(1,120)}=5.503,\ p=0.022$) (figure 4b).

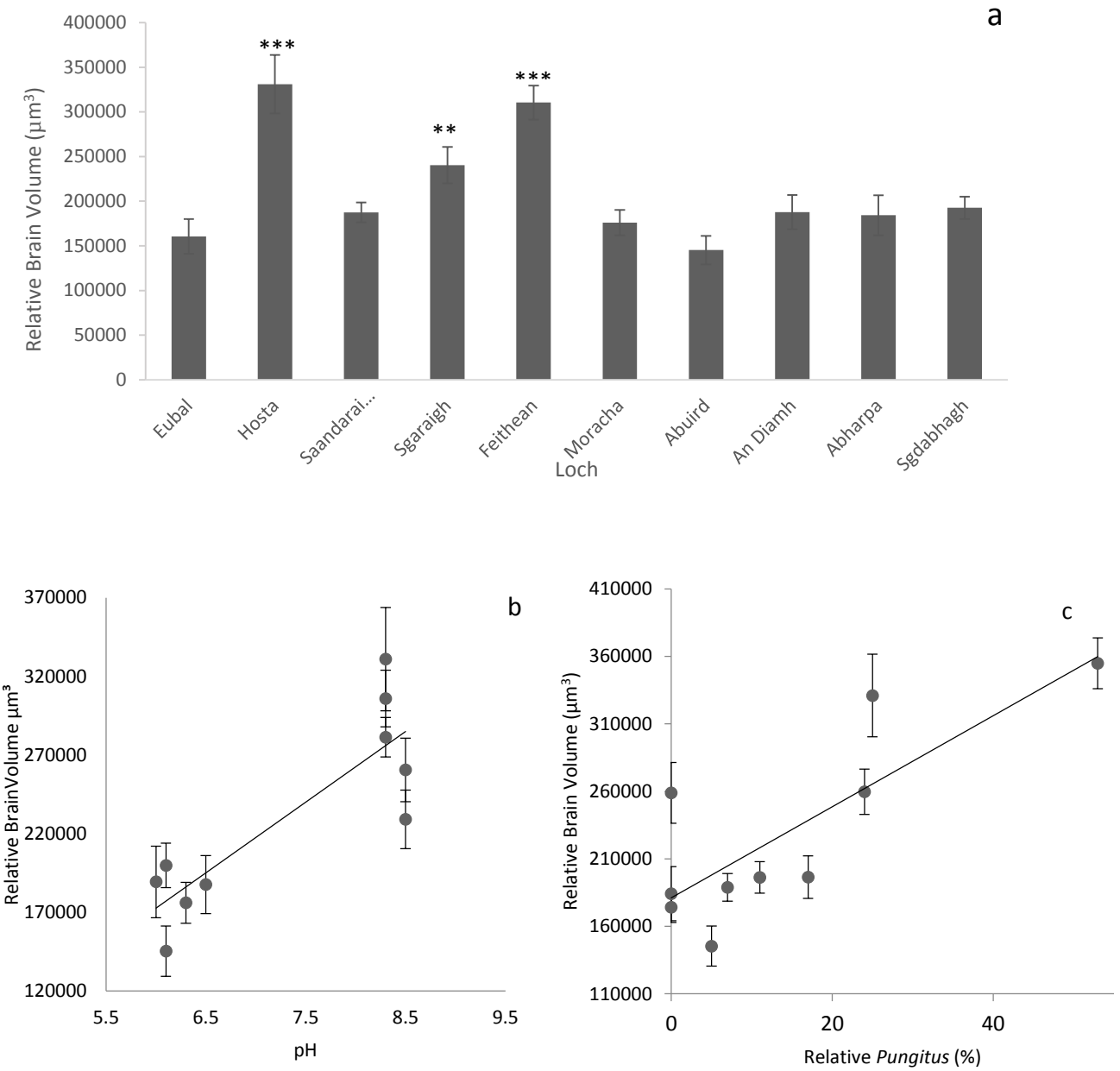

Figure 3(a). The mean ±SE 3-spined sticklebacks brain size sampled from different lochs of North Uist. Figure 3(b): Mean (±SE) Relative Brain Volume at differing pHs in Lochs of North Uist. Figure 3 (c): The mean ±SE relative brain volume and how it is affected by the relative abundance of 9-spined sticklebacks *Pungitus pungitus* in the environment. ***=p<0.01, ***=p<0.001.*

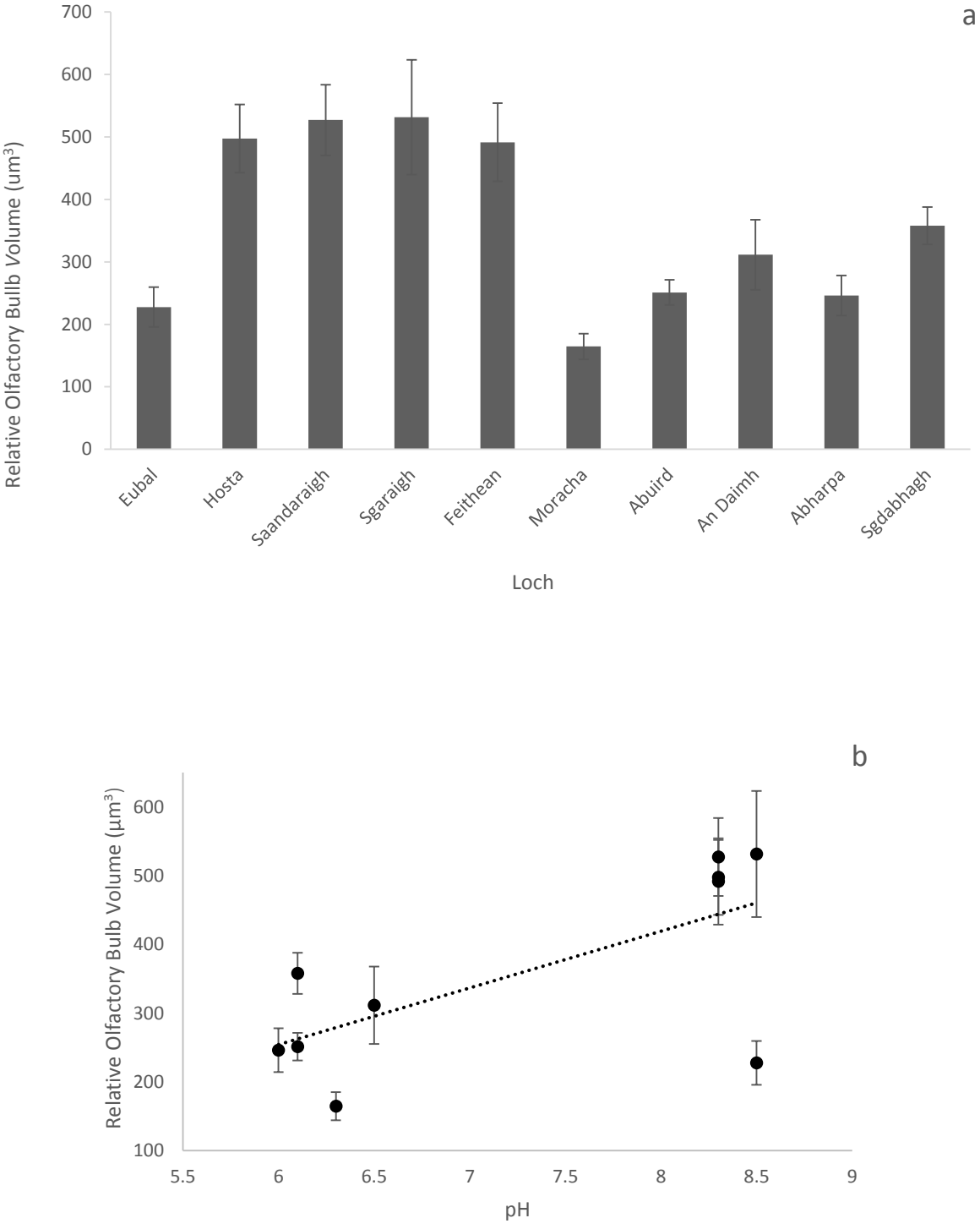

Figure 4(a): The mean ±SE relative olfactory bulb size from samples taken from different lochs of North Uist. Figure 4(b): The mean ±SE relative volume of the olfactory bulb of *Gasterosteus aculeatus* from lochs with differing pHs. Each point is the mean size for that pH and error bars = +/- 1SE. ****=p<0.001.*

5.1: Discussion

The purpose of this study was to investigate whether brain size and the size of individual brain regions could be affected by the environment. The results of this study indicate that brain size can differ between populations of the same species even within the same geographical location and these differences are driven by differing ecological and social variables within the environment of each population. In particular the relative size of the olfactory bulb was explained by water chemistry (ie. pH and Conductivity) and the relative size of the whole brain was explained by the percentage of 9-spined sticklebacks present in the environment in addition to pH and water conductivity. These findings support the theory that those regions of the brain that are more important in a particular environment develop more than those of less importance (Gonda, *et al.,* 2009a; Kihslinger and Nevitt, 2006; Kihslinger *et al.,* 2006; Lisney *et al.,* 2007)

The social brain hypothesis predicts that ability of an individual to use complex social information is dependent on its cognitive capacity, which is directly linked to brain size (Dunbar, 1988). Individuals with superior cognitive capacity should be able to keep track of more individual relationships and be able to respond appropriately during interactions with other individuals (Schultz and Dunbar, 2006). The relative size of the brain appeared to scale positively with the percentage of 9-spines in relation to 3-spines in the environment. It is predicted that as the two species shoal together but are reproductively isolated, more complex social interactions may arise from behaviours such as species recognition for mate choice.

The pH of each loch is a direct representative of the amount of dissolved minerals. In particular, in a study conducted by Spence *et al.* (2013) on the same populations in North Uist suggested that calcium concentration dictated the level of armor that a stickleback has. The fish in lochs with lower concentrations of calcium had lower armour levels and were shown to be less bold within their environment. It is therefore possible that the larger brain size of the fish found in more alkaline environments was a result of the increased confidence gained by having more body armour. For

example, in Kotrschal, *et al.* (2014) it was shown that the test subjects that were bolder, faster to habituate to, and more exploratory in open field tests were those individuals that had larger brains.

Other studies have shown positive correlations between the olfactory bulb and water turbidity (Huber, et al., 1997). Gonda *et al.,* (2009) suggested that an increase in the olfactory bulb may relate to lower visibility in the marine environment or a decrease may be due to reduced use of chemical signalling due to such factors as lack of predators or heterospecifics. The results of this study would suggest that it was the chemistry of the water in terms of pH that determined the size of the olfactory bulb rather than lower visibility. In terms of behaviour, Heuschele and Candolin (2007), Davison (2013) and Antoine, *et al.,* (2013) performed a mate choice experiment with 3-spined sticklebacks that showed olfactory signalling is boosted by the increase in pH.

It was unexpected that there was no difference in the size of the optic tectum between lochs. It might have been that the absorbance did not differ greatly enough between each loch (See Table 1) to elicit changes in the brain. Additionally, it must be recognised that rather than being turbid, the lochs were more tea-stained. The lack of differences between other brain regions was not surprising. Previous interspecific studies on fish have found positive correlations between telencephalon size and habitat complexity, as well as between olfactory bulb size and water turbidity (Huber *et al.,* 1997). In-depth anatomical analyses indicate that the telencephalon is involved in spatial learning (Broglio *et al.,* 2003). From the lack of differences in telencephalon size we can therefore predict that no loch was more or less environmentally complex than any other and that due to the lack of differences in cerebellum size, characteristics of the lochs did not act to effect motor function.

Anthropogenic induced climate change is ever changing the sensory environment of fishes (Donohue and Molinos 2009; Jeppesen *et al.,* 2010a; b; 2011). It has been demonstrated that the increased algal blooms as a result of climate change and anthropogenic practices reduce visibility (Utne-Palm, 2002; 2004) and the increased amount of photosynthesis increases pH due to the subsequent reduction in dissolved CO_2 levels (Heuschele and Candolin, 2007). From our work due to the increased size of the

olfactory bulb found in populations of sticklebacks found in lochs of higher pH (8.3+), it would be predicted that a shift towards a preference for chemical signalling will occur within the environment with associated changes in the size of the olfactory bulb.

Sexual dimorphism has been displayed in 3-spined sticklebacks during the nesting season (Kotrschal, *et al.*, 2012). It was shown that male three-spined stickleback have heavier brains than females, generated by the many cognitively demanding challenges that males face within this species, such as an elaborate courtship display, nest construction and a paternal care system (Kotrschal, *et al.*, 2012). One limitation of this study is that the sex of the specimens were not recorded and thus random sampling might have confounded the results by measuring more males than females in certain lochs, and vice-versa.

Another Limitation can be found in the use of 9-spined sticklebacks as a proxy for social complexity. Social complexity is made up of more factors than just the percentage of heterospecifics in the environment. It is a made up of a multitude of factors, for example in primates, brain size correlates with group size, range, and home range (Reader and Laland, 2002). It has been shown by Gonda, *et al.* (2011) that the number of interactions with conspecifics determines the size of the brain in 3-speined sticklebacks. However, these other measures of social complexity are very difficult to measure accurately in the field.

This study was a good starting point allowing an insight to be gained into how the environment shapes brain architecture in the field. However, while the analysis is more than simply a speculation it is however, difficult to assign causality on what drives these changes in brain architecture. Comparative studies such as this form the cornerstone of our current knowledge about brain size evolution, they are only correlative and therefore causations, such as diet, predation pressure and alike, are hard to prove with the approaches used. Therefore, the next stage of the study would be to rear sticklebacks in controlled conditions; to manipulate the sensory and social environment whilst controlling for all

other influences that is impossible to do in the field. Behavioural and cognitive testing before brain dissection and measurement would help to bridge the gap between brain and behaviour.

The correlation between brain and behaviour as a result of the rearing environment experienced throughout ontogenesis by the 9-spined stickleback *Pungitus pungitus.*

6.1: Introduction

It has been demonstrated in an array of studies that fish possess the ability to use either vision or olfaction for a wide variety of behaviours including mate choice, species recognition, shoaling and foraging. For example the Banded Kokpu *Galaxias fasciatus* show a distinct sensory bias for olfaction in search behaviour, even with one blocked nostril (Baker, *et.al.,* 2002). In *Rana cascardae* tadpoles, visual cues involved with kin recognition were not important; it was the olfactory signalling that was the key for this behaviour (Blaustein and O'Hara, 1982b; Waldman, 1985). In a species recognition experiment Kodric-Brown and Strecker (2001) found that *Cyprinodon maya* females identified conspecifics using both visual and olfactory cues equally but *C. labiosus* used solely olfaction. In a non-fish species, the hawk moth *Manduca sexta* showed a strong preference for vision over olfaction to find a nectar source when the two were decoupled (Goyret *et al.,* 2007). However, the results of a learning experiment demonstrated that *Spodoptera littoralis* moths have a very good capability to associate an odour with a reward (Fan, *et al.* 1997).

The reason for the variation in behavioural and sensory preferences across taxa is that behaviour is not fixed. Behavioural plasticity allows animals to develop behaviours that are adapted to the environment in which they find themselves and is often the first mechanism used to deal with environmental changes or stressors (West-Eberhard, 2003; Ghalambor *et al.,* 2007; Sih *et al.,* 2011), i.e. upon contact with an environmental stressor or novel environment, animals have the ability to adjust their behaviours proximately to lessen the negative effects of said environmental adjustment. Often these behaviours are influenced by experiences they receive in early life (Huntingford *et al.,* 1994). Thus the rearing environment has the potential to influence an individual's behavioural

phenotype, with individuals exposed to different types of environment developing different behaviours (Wiltschko *et al.,* 1987; Braithwaite and Guilford, 1995; Caldji *et al.,* 2000). For example Heuschele, *et al.,* (2009) demonstrated that Sticklebacks in turbid water used olfaction for mate choice whereas vision was more important in clear water. Indirect effects of increased turbidity are largely due to changes in the visual environment, through a reduction in the amount of light available and shifts in the colour of underwater light (Lythgoe, 1979; Seehausen and Schluter, 2003; Utne-Palm, 2002), reducing the visual distance and perception .

Changes in the sensory environment are able to influence animal communication by modifying how the signal is sent (e.g., endocrine disrupting chemicals altering the expression of sexually selected ornamentation; Baatrup and Junge, 2001), by altering signal transmission (e.g., noise pollution masking acoustic signals; Slabbekoorn and Peet 2003), or by affecting an animal's capacity to receive the signal due to shifts in receptor sensitivity (e.g. increased turbidity and changes to wavelength composition driving shifts in visual sensitivity; Hoffmann *et al.,* 2009).

The adaptationist model (Barton and Harvey, 2000) proposes that brains contain distinct regions that mediate associated sets of behaviour and thus selection for a particular series of behaviours should favour a change localized to the brain region that mediates it (Pollen, *et al.,* 2007). The idea that the environment experienced can modify brain structure and behaviour can be traced back to the 1890s (Kolb and Whishaw, 1998; Ramon y Cajal, 1928) and it was Hebb who made this a central feature of his neuropsychological theory (Hebb, 1949). For example, rodents have a profound sense of smell so it is not unreasonable to assume that experience would have substantial effects upon the olfactory system. The general conclusion is that deprivation of olfaction throughout development restricts the morphological development of the olfactory system, whereas olfactory enrichment results in greater development of this system (Doving and Pinching, 1973; Pinching and Doving, 1974; Rehn *et al.,* 1986). Other such studies showed differences in brain morphology as a result of the rearing environment with Gonda, *et al.,* (2011) showing that the size of all individual brain regions were smaller in

laboratory reared sticklebacks than ones taken from the wild, irrespective of population origin. It has been demonstrated in interspecific comparisons in primates that there exists a trade-off between visual and chemical centres of the brain (Barton and Harvey, 2000); this has also been demonstrated in Gonda*, et al.,* (2009a) where they found that fish housed alone in a simple environment developed greater olfactory bulbs and smaller optic tectum.

The significance of individual variability throughout ontogenesis as a source of adaptive fine-tuning, in particular environments, has been largely ignored. After hatching, fish larvae are visually orientated planktivores and grow over several orders of magnitude into their adult niches (Fernald, 1985; Kotrschal *et al.,* 1990). In the earliest developmental stages, teleost fish possess the smallest functional vertebrate brains, with extreme miniaturization of nerve cells. Allometric growth of the brain and its components then occurs throughout ontogenesis which lasts, on average for approximately 3-4years (Brandstatter and Kotrschal, 1989, 1990; Kotrschal, *et al.,* 1990; Toyoda and Uematsu, 1994). It is for this reason that 9-spined stickleback were chosen for this study.

It was Kolb and Whishaw (1998) who deduced that experience produces dissociable changes within the brain and these anatomical changes are associated with changes in behaviours. For example, rodents have incredibly effective olfactory systems so it is realistic to expect that experience would have substantial effects upon the structure of the olfactory system (Doving and Pinching, 1973; Pinching and Doving, 1974). It has been shown that olfactory deprivation leads to restricted morphological development of the olfactory system, whereas olfactory enrichment leads to enhanced development (Rehn *et al.,* 1986) altering the size and number of the associated neurons (Rosselli-Austin and Williams, 1990). It is expected that by changing the sensory environment to limit vision throughout ontogenesis by rearing groups of 9-spined sticklebacks in both clear and ink-black water, a reduction in the size of the optic tectum and an increase in the size of the olfactory bulb will be seen in the fish reared in the dark water environment compared to fish reared in a clear water environment. This will then act to affect sensory preference for shoaling tested through a dichotomous choice design

where vision and olfaction are presented diametrically at different ratios. There is a vast amount of evidence to suggest that in social fish, when placed individually in an unknown environment will join other conspecifics and, if given the choice of shoals of varying sizes, will exhibit preference for the largest one (Prichard*, et al.,* 2001; Agrillo and Dada, 2007; Agrillo*, et al.,* 2009). This tendency to shoal with the larger group was exploited in this experiment.

The numerical cognition has the potential to enhance survival and reproduction among species (Agrillo, *et al.,* 2011). For example, fish that shoal in order to reduce the risk of predation, benefit from choosing larger shoals (Barber and Wright, 2001). It was Agrillo, *et al.,* (2008a; b; 2009) and Buckingham, *et al.,* (2007) who concluded that it was the ratio of individuals that was the factor in determining the larger shoal and there was a threshold where the fish could no longer determine the larger shoal. In their studies, they used ratios of between 3:1 and 4:5. Numerosity is a good test of sensory capacity because it allows us insight into which sensory mechanism is favoured in decision making. However, it has yet to be explored whether there is a preference for vision or olfaction in numerical cognition and whether this difference is related to the size of the associated brain regions. It is predicted that sticklebacks reared in visually limited environments will show a preference for olfactory cues when determining the larger shoal. It is also predicted that as the ratio of shoal sizes gets closer to 1 fish will begin to show no preference with number of switches, time taken to shoal increasing with difficulty and time spent with initial choice decreasing. It is the aim of this study to explore how the environment experienced throughout ontogenesis affects the sensory regions of the brain and what effect this has on visual and olfactory preferences for determining shoal size at different ratios.

7.1: Method

7.1.1: Test Subjects

One hundred and twenty 9-spined sticklebacks were collected from the field and reared in captivity for 6 months. They were caught using large hand nets on the 8th October 2013 from Yewthorpe Beck in Gainsborough, Lincolnshire, DN21. The fish were divided into 6 replicate tanks: 3 clear water and 3 ink-black water treatments created through the use of Pond Dye: Dyofix Pond Black, where 0.16g of powder was added per litre of water to restrict light to a depth of 2cm measured using a white ruler. Pond Dye was used as a method for ethically limiting the visual environment. It is designed for colouring the water of fish tanks and is extremely safe for fish with no suspended particles or toxic chemicals. Pond Black absorbs and blocks all daylight, allowing no light to penetrate below the surface thus restricting the visual environment. Each tank was opaque and had a capacity of 45L. The temperature of each tank was maintained at 12°C (+/- 1) by a uniquely designed closed flow-through water system that relied on heat exchange. In each tank 2m of copper pipe was cut into 4 sections, connected by hose pipe and wrapped in electrical tape. The system connected all 6 tanks and to one-another and then terminated at a water cooler, where the system started again. The fish were fed on a diet of 1.5 diced bloodworm cubes per tank and fed on a 5 day on 2 day off cycle. Cleaning and 1/3rd water changes occurred on Tuesdays and Fridays as not to stress the fish out too much. The light cycle in the room was consistent with that of actual day length in order for the individuals to maintain a natural circadian rhythm and allow natural growth. The fish were kept in these conditions for 6 months (see Figure 5).

Figure 5: Housing design for the test subjects in their individual tanks

7.1.2: Experimental Setup

The dichotomous choice set up and tank design used to assess shoaling preferences was similar to those used in other studies (Krause, 2002; Barber and Wright, 2001; Wong and Rosenthal, 2005; Agrillo, *et al.,* 2008a;b). Experiments were carried out in a large rectangular glass tank (65x38x40cm) with two smaller tanks at either end. The larger central section, the 'choice arena' housed the test subject. The outer tanks (7x25x45cm) housed the stimulus fish. Water was at a depth of 7cm and no water exchanges occurred between containers as the chemical environment needed to be controlled. A 7.5 cm preference zone parallel to the shoal containers, approximately 3 body lengths (Pitcher, 1979), was marked on the walls of the test tank using a black marker. A fish was considered in the preference area when any part of its body crossed the line. The tank was placed on a white surface to enhance contrast for observation purposes and the outside of the tanks covered in black plastic to prevent any fish from using cues from outside the arena (Agrillo, *et al.,* 2009). The odour stimulus was created by housing 10 fish in 1 litre of oxygenated water for 48hrs prior to experimentation to create the raw stimulus water, which was then diluted accordingly to match the ratios of the visual stimulus

(Table 2) by pipetting the required amount of raw stimulus into a beaker and topping up with water to 50ml. This was to ensure the odour stimulus received was the same each time for every fish but at different concentrations to give the impression of differing shoal sizes. This method was chosen from a series of successful pilot studies carried out prior to this experiment.

Odour	Dilution (50ml Burette)
10 fish	no dilution
8 fish	40ml stimulus: 10ml water
7 fish	35ml stimulus: 15ml water
6 fish	30ml stimulus: 20ml water
5 fish	25ml stimulus: 25ml water
4 fish	20ml stimulus: 30ml water

Table 2: Fish odour dilutions for chemical stimulus.

Fish were kept in their rearing tanks up to 48hours prior to experimentation. At this time they were transferred into holding tanks containing clear water. These tanks were oxygenated and maintained at 12°C. The holding tanks were labelled with all the information about the rearing environments.

The time of day that each subject was tested was accounted for buy using a number table. Each fish was labelled 1-6 and this was the order of which they were they received one trial (ie. one trial and then a five trial break) The numerical trial ratio was randomised for each fish but they only received a certain trail ratio once.

7.1.3: Control Experiments

These were designed to show that through being given an isolated stimulus, the fish from both treatments still maintained the ability to use either olfaction or vision to determine shoal size.

Individuals were randomly selected from each treatment across the 6 tanks for the control experiments. Six fish (one from each tank) were tested over 2 days with a total of 12 fish being tested per week. For the visual trials fish were presented with two shoals, one in each stimulus tank, with ratios of 10:4(0.4), 8:4(0.5), 7:4(0.57), 6:4(0.67) and 5:4(0.8) (Agrillo, *et al.,* 2008b; 2009; 2011; Buckingham, *et al.,* 2007). This design was repeated for the olfactory trials just with odour being dripped in through a burette at a rate of 10ml/min. Each fish was numbered 1-6, which denoted its order of trailing. The ratio, stimulus type, side of largest shoal was selected at random using a table generated in Excel. Each test subject was placed into an opaque, open ended holding container in the centre of the arena. After 1 minute of acclimatisation the container was lifted, stopwatch started and the test subject was given 5 minutes within the arena, with their location recorded every 10 seconds in terms of which zone it was in. Zone 1 was always the side of the smaller shoal and Zone 4 was the side of the larger shoal. In addition to this, first choice, time spent at first choice, number of switches and time taken to enter a choice zone were all recorded for each fish. First choice was the preference zone (Zone 1 or 4) in which it swam into first. Time spent at first choice was the amount of time(s) that it spent in the initial preference zone before leaving. Time taken to enter a zone was the amount of time in which it took the individual to enter either preference zone after release from the holding container. The number of switches was calculated from the amount of times a full length of the tank from preference zone 1 to 4 occurred. If the test subject did not enter the other preference zone before returning, this did not count as a switch.

7.1.4: Sensory preference trials

The remaining 40 individuals were trialled (20 dark and 20 clear water fish) with a similar set up as the control trials. Each individual received both visual and olfactory stimulus diametrically and simultaneously (figure 6) at ratios of 2.5:1(0.4), 2:1 (0.5), 1.5:1(0.67), and 1.25:1(0.8), where the smallest shoal remained constant at 4 individuals. Six fish were tested per day with a total of 18 being

tested per week. One individual was selected from each tank at random and given a number between 1 and 6 which denoted the trial order. The ratio and stimulus side was selected at random using a table generated in Excel but each fish only received a given ratio once. Each fish was placed in an opaque releasing pot for 1 minute prior to the trial for acclimatisation. Each test subject was given 5minutes within the arena and their location recorded every 10 seconds by the zone in which it was residing. Zone 1 was always the side of olfactory preference and zone 4 was the side of visual preference. In addition to this, identically to the control trials (section 7.1.3), first choice, time spent at 1st choice, number of switches and time taken to enter a choice zone were all recorded for each fish. Sensory preference score was generated by recording the location of the fish every 10 seconds for 300 seconds. + 1 for every 10 second point the test subject was in the olfactory zone (Zone 1) and -1 for every 10 second data point for in the visual zone (Zone 4). +30 would be complete olfactory preference and -30 would be complete visual preference with 0 denoting no preference.

After each fish had been given all trials the protocol of euthanasia and preservation was followed identically to that of section 3.1.2 for both control and test fish.

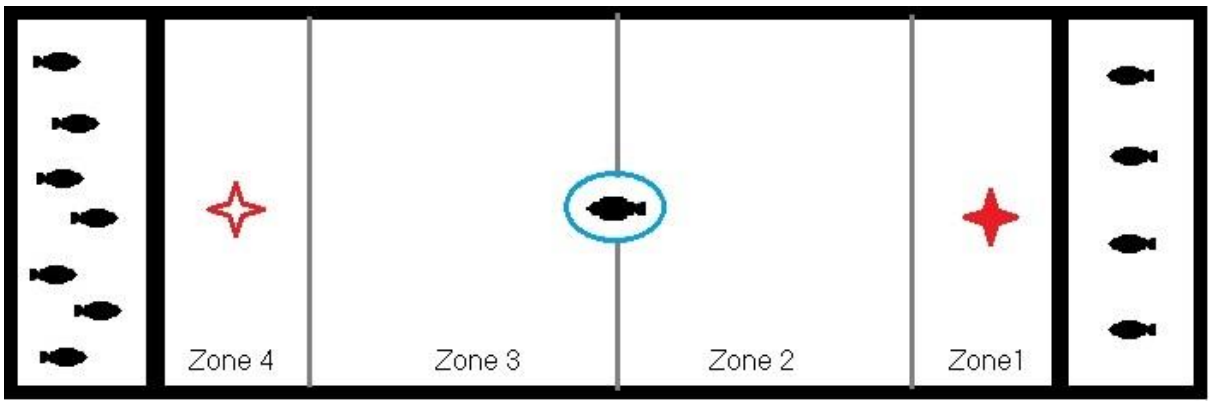

Figure 6: The tank design for the final experiment. Fish at either end are stimulus fish, the blue circle represents release pot. Stars represent odour stimuli

7.1.5: Brain Dissections

After fixation in formalin solution all the specimens were labelled with an assigned fish ID number based upon the rearing tank, treatment type and test number. Standard length, defined as the distance from the tip of the upper jaw to the end of the vertebral column, was measured to the nearest 0.01mm using ImageJ software after a photograph was taken of the specimen under an imaging microscope. Specimens were placed under a dissecting microscope, and their brains were extracted dorsally after removing the parietal, frontal and nasal bones (Park and Bell, 2010). Digital images of the dorsal and lateral aspects of the brain were taken using a computer-assisted video image analysis system, Motic Images 2.0. Images were always 800x600pixels, magnification 8 and were saved in JPeg format.

The length (L), width (W) and height (H) of all brain regions were measured to the nearest 0.01μm using ImageJ software (see below). Height and width was measured as the greatest distance perpendicular to the brain and length was measured as the greatest distance parallel to the brain. The volume (V) (μm^3) of each brain region and absolute brain size was calculated using the ellipsoid model (Pollen, 2007; Gonda *et al.,* 2009, 2010, 2011, 2012):

$$V= (L * W * H)/6.$$

Following that, the relative brain size was calculated by dividing the absolute brain size by the standard length which had been converted to μm.

7.1.6: Data Analysis

All data was checked for normality using the Kolmogorov-Smirnov Test and the Shapiro-Wilk Test and those data that were deemed not normally distributed ($p<0.05$) were log10 transformed. The absolute brain sizes were compared between treatments using the Student's independent t test. The standard length and each individual brain region (absolute and relative size) were compared between groups

with a bonferroni corrected *p* value of 0.01 (brain size only) using the Student's independent t test. Analysis of all trials were carried out using a GLMM with treatment and trial ratio as fixed factors and preference score, time until shoaling, time at 1st choice and number of switches as the dependant variables and tank number as a random factor. If Maunchly's test showed that sphericity had been violated then the most appropriate elipson was used depending on if the value was above or below 0.75. The interaction was assessed to see how the impact of one variable (eg. Numerical trial ratio) depends on the level of the other variable (treatment group). For assessment of 1st choice data, it was analysed using binomial tests.

8.0: Results

8.1.1: Fish Length

Fish Length did not differ significantly between treatments *(t=-0.766, df=38, p=0.448).*

8.1.2: Brain Size

The relative and absolute sizes were measured but the results were consistent with one another so only the absolute sizes results are presented here. The absolute sizes of the entire brains did not significantly differ between groups *(Mean ± SE,* Clear water*: 14.13mm³ ±1.27;* Dark water*: 14.75mm³ ±1.32; t=-0.342, df=38, p=0.734).* Absolute size of the olfactory bulb were significantly bigger on average in fish who underwent the dark water treatment *(t=-3.949, df= 38, p<0.001*) compared to the clear water treatment. Size of the optic tectum from fish of the dark water treatment was significantly smaller *(t=3.677, df=38, p=0.001)* when compared to the fish reared in the clear water treatment, with no difference elicited between any other region of the brain *(Telencephalon: t=1.34, df=38, p=0.894; Cerebellum: t=-0.505, df=38, p=0.616; Dorsal Medulla: t=-0.446, df=38, p=0.658)* (Figure 7).

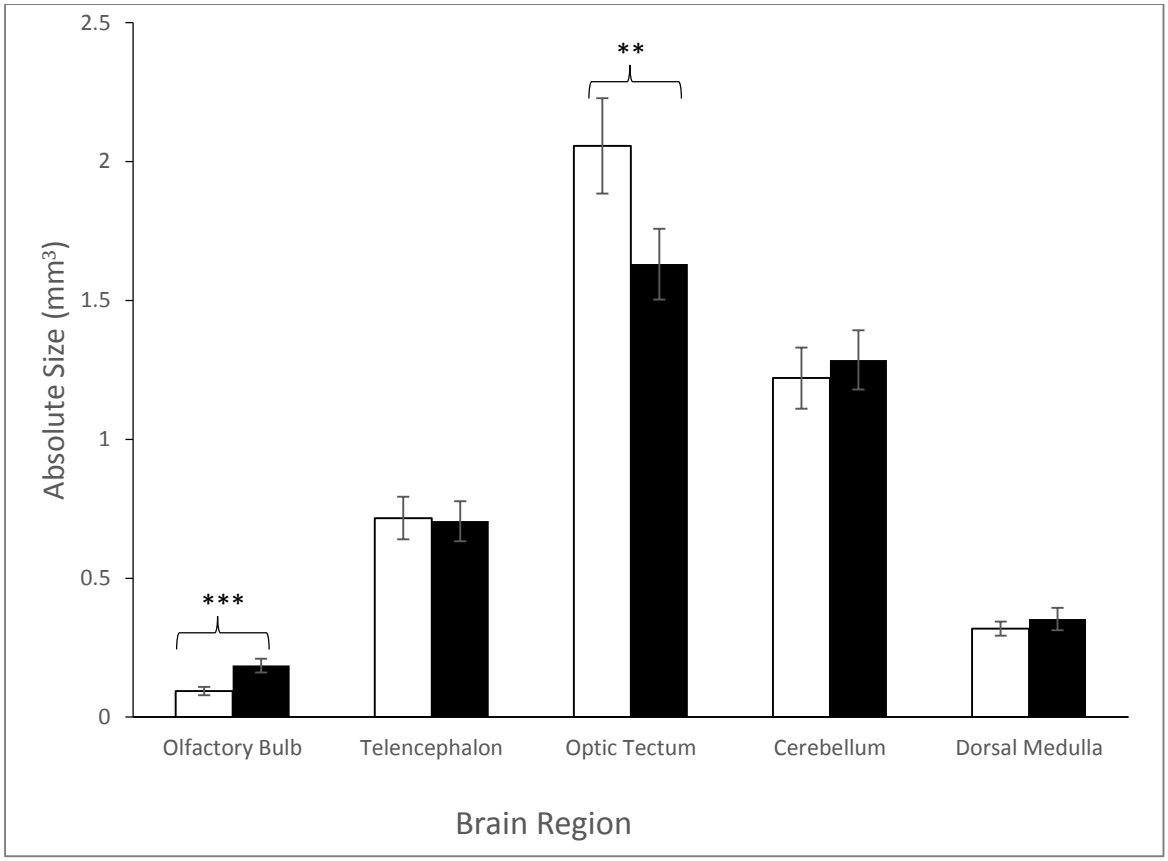

Figure 7: The mean ±SE absolute sizes of each brain region from 9-spined sticklebacks reared in dark (black bars) and clear water (white bars) treatments. Asterisks represent values that are significantly different from one another. ***=p<0.01, ***=p<0.001.*

8.2: Control Behavioural Experiments

8.2.1: Olfactory Based Trials

Tests of within subjects effects showed that there was a significant effect of the trial ratio on preference score *($F_{(2.674,\ 48.132)}=4.083,\ p=0.014$)* but the treatment had no significant effect *($F_{(1,18)}=2.154,\ p=0.159$*) nor was there any interaction *($F_{(2.674,\ 48.132)}=0.799,\ p=0.488$)* (Figure 8). Binomial tests show that for the olfactory controls as a whole, there was a significant first choice preference for the larger shoal (Zone 4) by fish from the clear water treatment *($N_1=35\ N_2=10,\ p<0.001$)* and from the dark water treatment *($N_1=34\ N_2=11,\ p=0.001$)* (Figure 9a;b). There was no significant effect of numerical trial ratio on the time until 1st choice was made *($F_{(1,80)}=5.916,\ p=0.06$)* or the time

spent with the 1st choice *($F_{(1,80)}=2.128, p=0.149$)*. Additionally, there was also no significant effect of treatment group on the time until 1st choice was made *($F_{(4, 80)}=1.589, p=0.442$)* or the time spent with the 1st choice *($F_{(4,173)}=1.905, p=0.118$).*

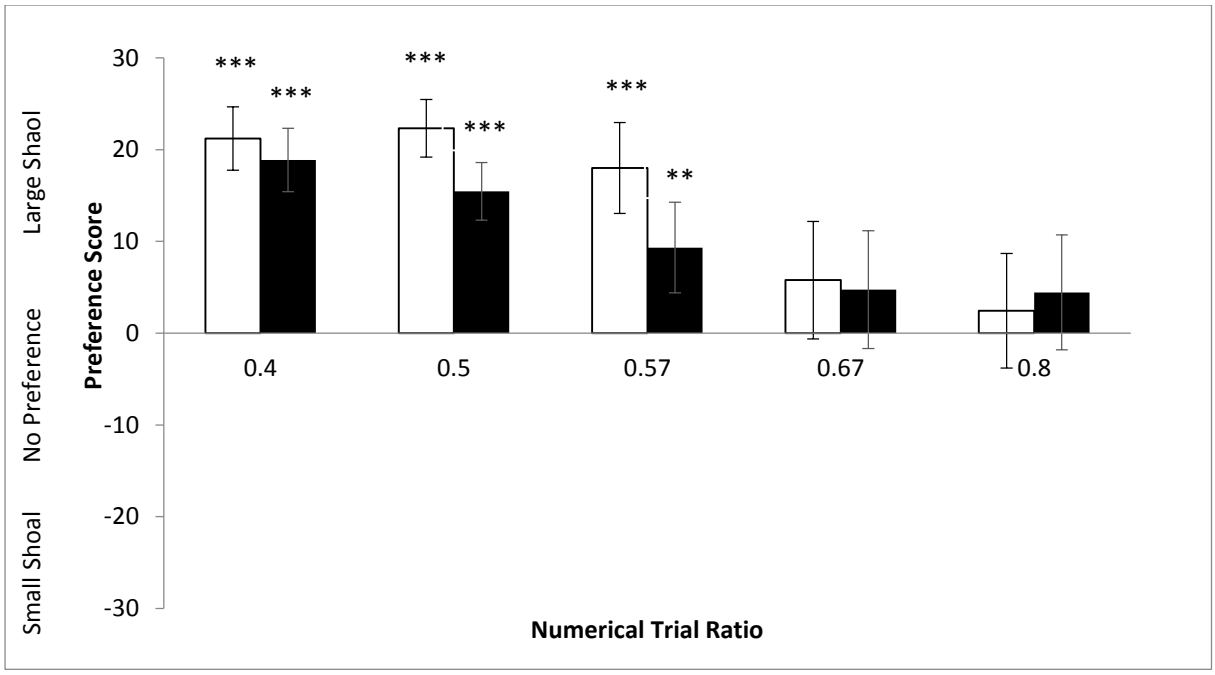

Figure 8: The average ±1SE preference score of dark and clear water reared sticklebacks at different shoal size ratios presented in the form of olfactory stimuli. White bars represent the clear water treatment and the black bars represent the dark water treatment. * represent data that is significantly different from 0. ***=p<0.01, ***=p<0.001.*

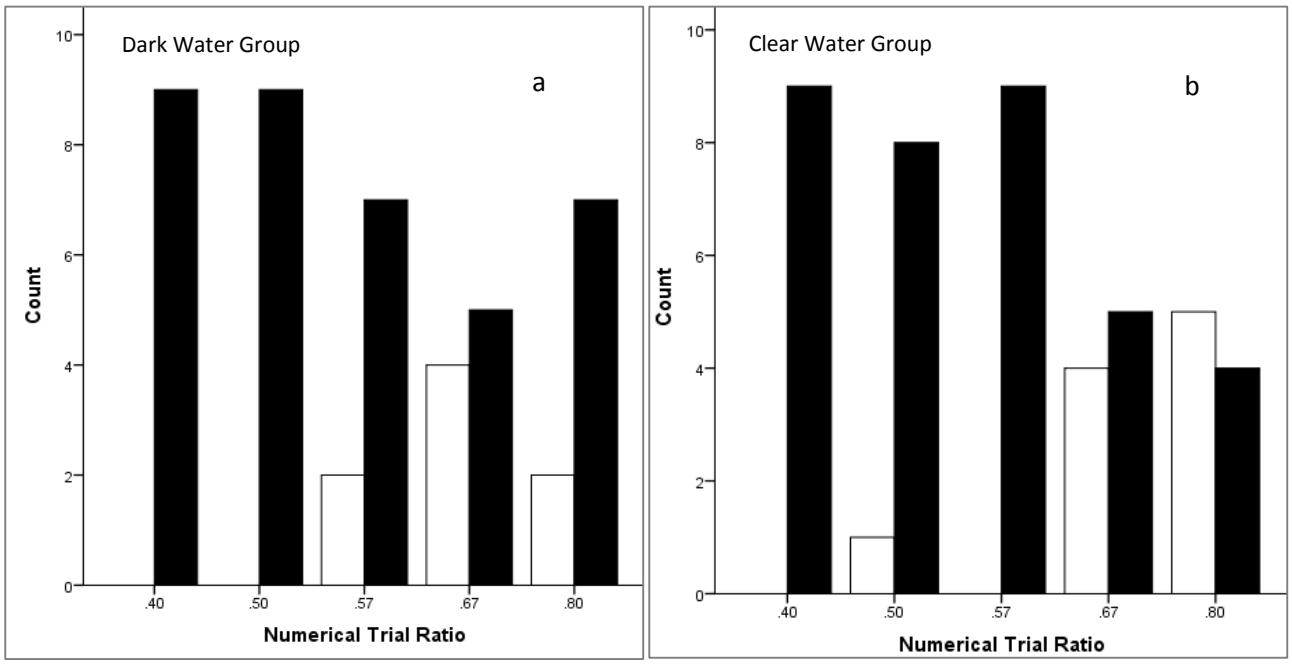

Figure 9(a): The number of times zones 1 (small shoal) and zone 4 (large shoal) was first choice in the olfactory control trials at each numerical trial ratio for fish reared in dark water. Black bars represent the large shoal and the white bars represents the small shoal. Figure 9(b): The number of times zones 1 (small shoal) and 4 (large shoal) was 1st choice in the olfactory control trials at each numerical trial ratio for fish reared in the clear water treatment. Black bars represent the large shoal and the white bars represent the small shoal.

8.2.2: Visual Based Trials

It was shown that there was a significant effect of the trial ratio *($F_{(2.364, 38)}=5.886$, $p=0.004$)* and the and the treatment *($F_{(1,18)}=16.091$, $p=0.001$)* on preference with a non-significant interaction *($F_{(2.364,38)}=1.184$,$p=0.321$).* With a Bonferroni corrected *P* value of 0.01 the only significant difference between groups was at a ratio of 0.67 *($t=3.014$, $df=18$, $p=0.007$)* (Figure 10). Binomial tests show that for the visual controls as a whole, there was a significant first choice preference for the larger shoal (Zone 4) by fish from the clear water treatment *($N_1=37$ $N_2=8$, $p<0.001$)* and from the dark water treatment *($N_1=36$ $N_2=9$, $p<0.001$)* (Figure 11a;b). There was no significant effect of numerical trial ratio on the time until 1st choice was made *($F_{(1,80)}=1.046$, $p=0.31$)* or the time spent with the 1st choice *($F_{(1,80)}=3.680$, $p=0.059$)*. Additionally, there was also no significant effect of treatment group on the time until 1st choice was made *($F_{(4, 80)}=1.666$, $p=0.167$)* nor time spent with the 1st choice *($F_{(4,173)}$*=1.337, *$p=0.23$).*

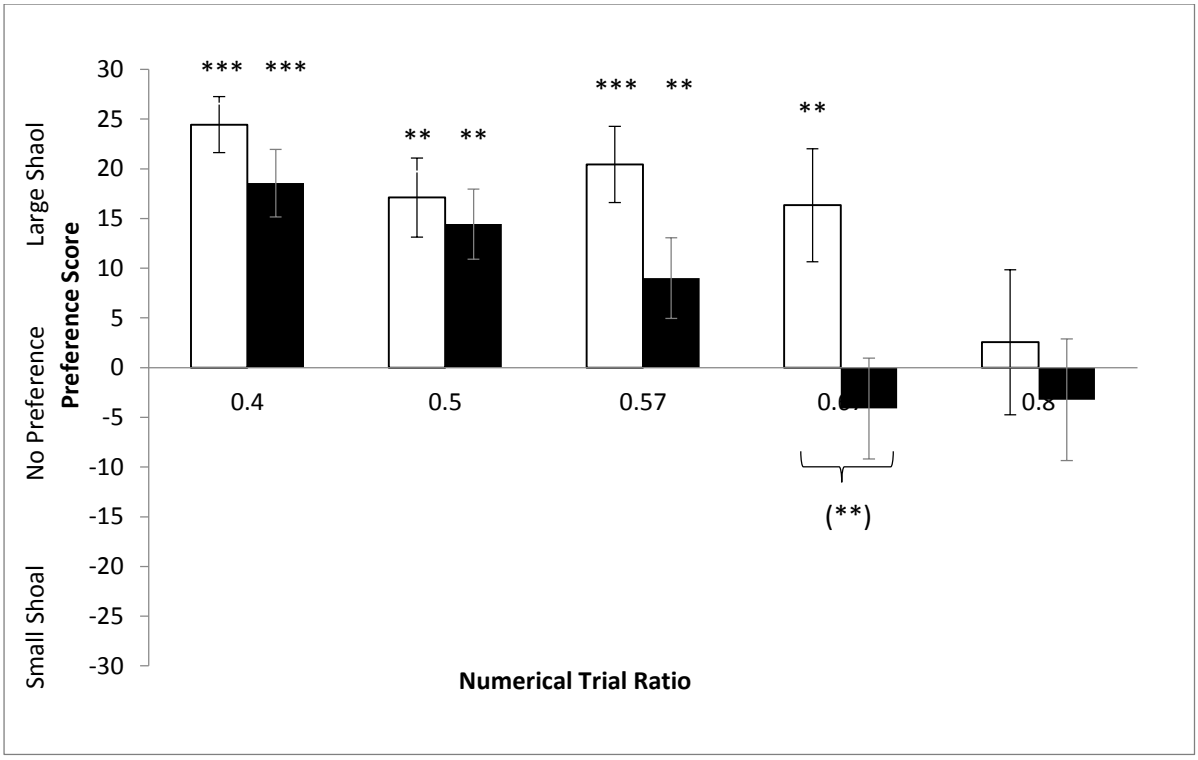

Figure 10: The mean ± 1SE preference score of dark and clear water reared sticklebacks at different shoal size ratios presented in the form of visual stimuli. White bars represent the clear water treatment and the black bars represent the dark water treatment. Top * represent data that is significantly different from 0. And bottom (*) represent data significantly different from one another. ***=p<0.01, ***=p<0.001.*

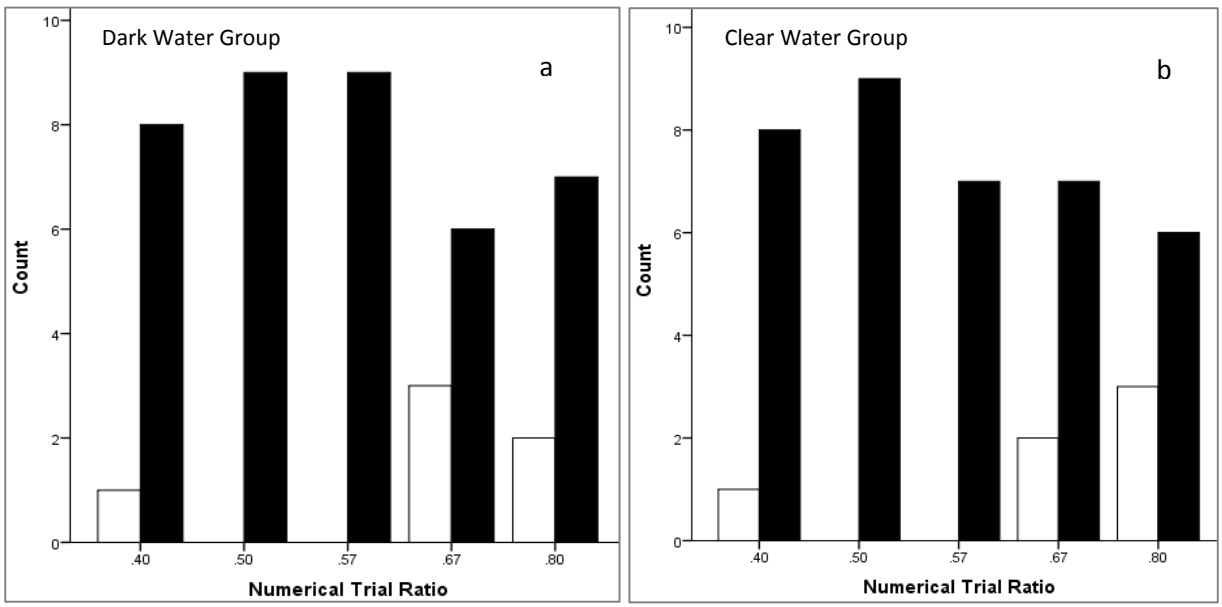

Figure 11(a): The number of times zones 1 (small shoal) and 4 (large shoal) was first choice in the visual control trials at each numerical trial ratio for fish reared in the dark water treatment. Black bars represent the larger shoal and the white bars represent the smaller shoal. Figure 11(b): The number of times zones 1 (small shoal) and 4 (large shoal) was first choice in the visual control trials at each numerical trial ratio for fish reared in the clear water treatment. Black bars represent the larger shoal and the white bars represent the smaller shoal.

8.3: Sensory Preference and Brain Size

8.3.1: Sensory preference trials

Tests of within subjects effects showed that there was no significant effect of the trial ratio on treatment *($F_{(2.887, 38)}=1.70, p=0.175$)*, interaction *($F_{(2.887, 38)}==0.680, p=0.561$)* or preference score *($F_{(1,38)}=1.304, p=0.262$).* A series of independent t-tests with a Bonferroni correction giving a *p* value of 0.01 suggests the one significant difference was between preference scores at the ratio 0.5. *($t=-2.711, df=38, p=0.001$).* Binomial results show that as a whole there was a significant preference for Zone 1 (olfaction) as the first choice for fish from the dark water *($N_1=66\ N_2=31, p<0.001$)* and Zone 4 (vision) for fish of the clear water treatment *($N_1=33\ N_2=63, p=0.04$)* (Figures 12 and 13). There was no significant effect of numerical trial ratio on the time until 1st choice was made *($F_{(1,173)}=7.05, p=0.102$)* or the time spent with the 1st choice *($F_{(1,173)}=1.589, p=0.209$)*. Additionally, there was also no significant effect of treatment group on the time until 1st choice was made *($F_{(4,173)}=0.625, p=0.645$)* or the time spent with the 1st choice *($F_{(4,173)}=1.678, p=0.157$)* (Figure 14).

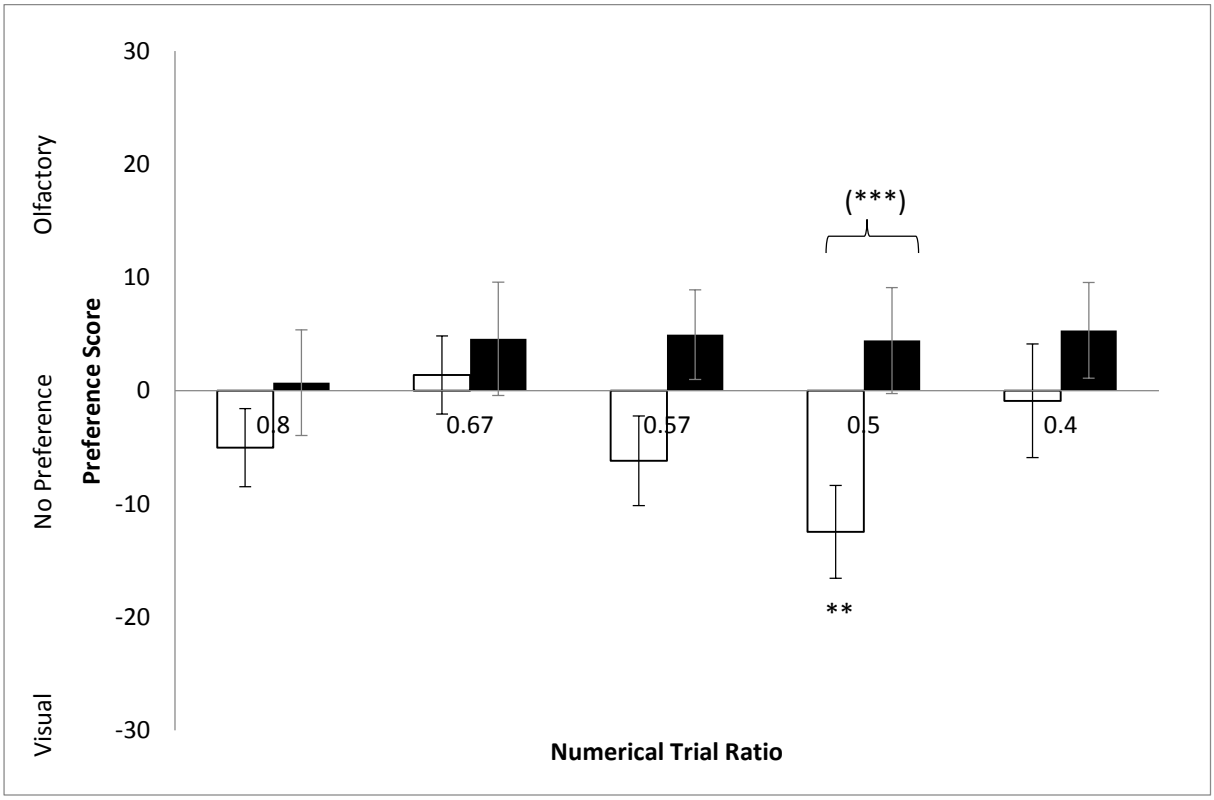

Figure 12: The mean ± 1SE preference score of dark and clear water reared sticklebacks at different shoal size ratios presented in the form

of visual stimuli against the corresponding olfactory stimuli where +30 is olfactory preference and -30 is visual preference. Black bars represent fish from the dark water treatment and white bars represent fish from the clear water treatment. Bottom * represent data that is significantly different from 0 and top (*) represent data significantly different from one another. ***=p<0.01, ***=p<0.001.*

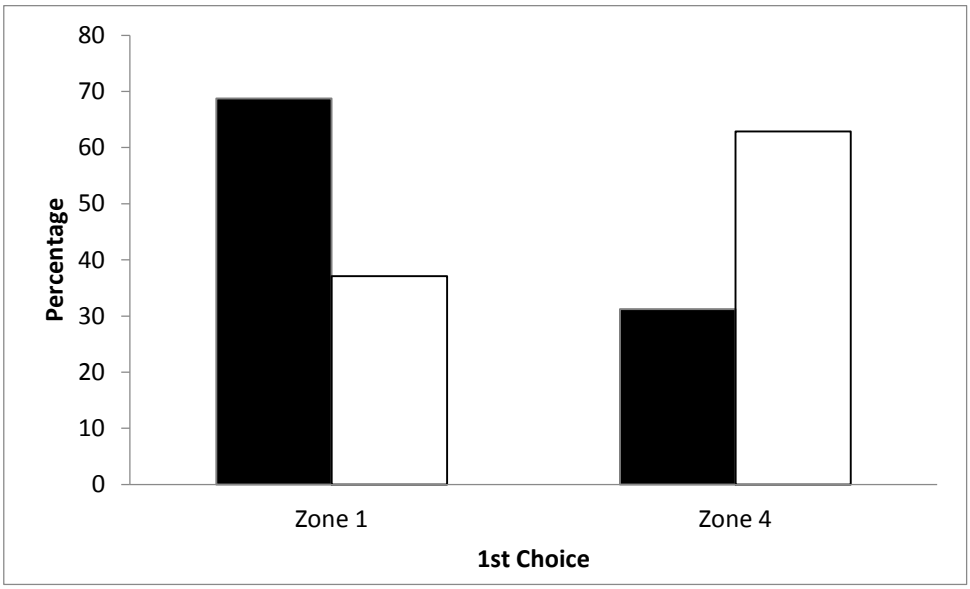

Figure 13: The percentage of fish that as a whole chose Zone 1 (olfactory preference) and Zone 4 (visual preference) throughout all trials. Black bars represent fish from the dark water treatment and white bars represent fish from the clear water treatment

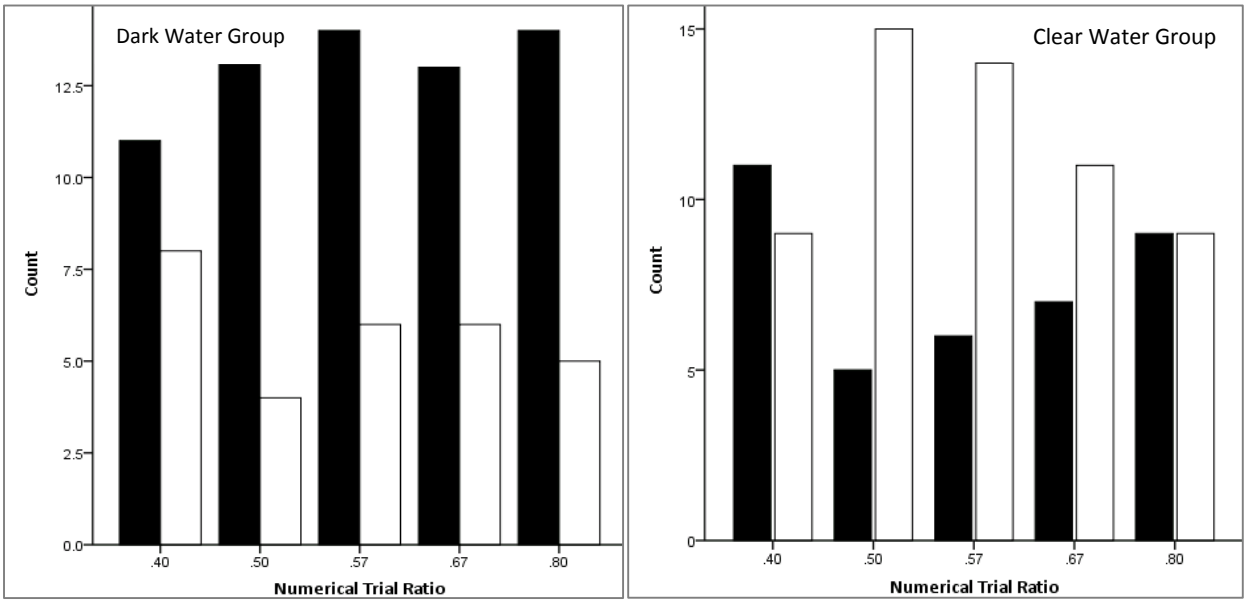

Figure 14(a): The results for number of times zones 1 and 4 were first choice at each numerical trial ratio for fish reared in dark water. Black bars represent zone 1 (olfactory preference) and the white bars represent zone 4 (visual preference). Figure 14(b): Percentage of times zones 1 and 4 were first choice at each numerical trial ratio for fish reared in dark water. Black bars represent zone 1 (olfactory preference) and the white bars represent zone 4 (visual preference).

9.1: Discussion

The aim of this study was to manipulate the sensory environment of 9-spined sticklebacks in order to elicit change in the associated sensory brain regions and examine what effect this has on cognition and behaviour. Previous studies which have explored the links between behavioural plasticity and brain size have often been carried out using comparative methodology (Gonda, *et al.,* 2011; Lefebvre *et al.,* 1997; Pollen, *et al.,* 2007). This study took a more causal approach in that all environmental factors were controlled such as group size, diet, feeding regime, temperature, and only was the sensory environment manipulated.

It was shown that the fish reared in the dark water developed brains with larger olfactory bulbs and smaller optic tecta than fish reared in the clear water environment, with all other brain regions showing no difference between them. The standard length did not differ between the two groups nor did absolute brain size. Therefore we see that deprivation of the visual environment led to a reduced development of the visual system and enhanced development of the olfactory system. Similar trends have also been displayed in rodents where deprivation of olfaction throughout development restricted the development of the olfactory system (Doving and Pinching 1973; Pinching and Doving 1974; Rehn, *et al.,* 1986). As seen in the previous chapter, social complexity increased absolute brain size in wild sticklebacks. The fact that there was no difference between absolute brain sizes in this experiment suggested that the complexity of the social environment had not been changed. In addition, the lack of difference between standard length and all other brain regions except the olfactory bulb and the optic tectum confirms that only the sensory environment was manipulated. The results of this study are in accordance with that of Huber and Rylander (1992) which found that species of minnows differed in their sensory modalities which correlate with the physical parameters within their environment and this was reflected in the size of the associated neural structure, i.e. Fish in clear water environments had larger optic tectum and the olfactory bulb was larger in fish species who preferred turbid environments.

Even though these differences in brain size were identified and half the fish had been reared in a visually deprived environment, the dark water fish still retained the ability to use vision as well as olfaction to determine the larger shoal up to a ratio of 0.67 where at this point they either couldn't determine which was the larger shoal or the difference in the size of the shoals were at a point where it did not matter to the fish. The same goes for the clear water fish, except the data showed that they were better at using vision to determine shoal size in the visual controls than the dark water fish. It was predicted that the number of switches, time taken to choose a shoal would increase and time spent at first choice would decrease with increased difficulty of the trial (numerical ratios closer to 1). In fact, there was no difference in any of those for any trial, which suggests that they were approaching each trial with the same dynamism.

Because in the previous trials, it was shown that fish from both groups could use both vision and olfaction and that they had a tendency to shoal with the larger shoal, vision and olfaction were put against one another diametrically to test sensory preference. In the final experiment, neither dark nor clear water reared fish showed any deviation from chance and thus showed no preference for either vision or olfaction for shoaling at any ratio. However, when the data was examined further in terms of 1st choice, it was seen that the dark water fish showed an affinity towards the olfactory stimulus and the clear water group showed an affinity for the visual stimulus. This shows that the behaviour had in fact deviated in relation to the associated sensory brain region. It could have been that the fish in fact had too long in the choice arena that after the initial choice was made, they had the confidence to explore the environment thus averaging out the time spent in each preference zone and that 1st choice could be more of an appropriate measure to determine preference as it shows us which senses the fish are using to initially make snap decisions as to which group to shoal with.

Two anomalies do exist within the clear water data. The first is at the 0.4 ratio of the final trial. It appears that a trend might be starting to form especially with the previous result (0.5) being significantly towards the visual stimuli. It is possible this has arisen due to the amount of stimulus

within the tank at 0.4 making the fish either confused or feel like it didn't matter which group the shoaled with as they always felt like they were with the bigger shoal. This is also shown in Figure 9c where at the ratio of 0.4, the fish showed an equal preference for vision and olfaction for 1st choice.

Much like the first chapter, the sex of the subjects was not accounted for. This causes limitations to the study in that is had been shown by (Herczeg *et al.,* 2014; Kotrschal, *et al.*, 2012). that during the nesting season there is sexual dimorphism in brain size. However, this did not appear to have an apparent affect on the results of the study.

To conclude, the environment that the fish experienced throughout ontogenesis was effective in manipulating changes in the sensory regions of the brain and these changes elicited differences in sensory preference. In other words, fish reared in the dark environment developed bigger olfactory and smaller optic regions than the clear water group and this altered the behaviour in that they used olfaction as their initial mechanism to determine shoal size as opposed to vision which was apparent in the clear water group.

This study has successfully provided experimental evidence that the environment experienced throughout ontogenesis does act to manipulate brain architecture and this does have effect on behaviour in terms of sensory preference for communication and decision making. Additionally sensory preference in numerosity has never been explored.

10.1: General Discussion and Conclusions

This study has provided further evidence for brain plasticity occurring in wild populations. It has demonstrated that variation in brain architecture occurs due to the variation in the social and chemical environment. This is augmented by the lab based experiment carried out that showed significant differences in the size of the visual and olfactory regions of the brain when reared in dark water as compared to clear water. This study has shown that changes in the size of brain regions

does have behavioural effects in that fish with larger olfactory bulbs show an affinity towards using olfaction as the primary method of decision making and those with larger optic tecta showed an affinity towards using vision as the primary method.

The first part of this study was key in allowing the exploration of whether brain size varies between populations of the same species. It was found that sticklebacks reared in more socially complex environments developed bigger brains relative to their body size. It was also shown that fish in more alkaline environments developed bigger olfactory bulbs as a result, thus showing us that the environment does impact upon brain morphology with more important regions developing more than those of less importance. Following from this, the lab based experiment allowed for manipulation of only the sensory environment with every other parameter apart from sex being controlled for. It was shown that the fish reared in dark water developed brains with much larger olfactory bulbs and much smaller optic tecta than those from the clear water treatment. The fish still retained the ability to use vision and olfaction in the control trials but the individuals of the clear water treatment appeared to have greater capabilities in the visual trials. In the diametric trials there appeared to be no preference for any either stimuli but under closer inspection, it was found that in terms for first choice, dark water fish has an affinity towards the olfactory stimulus and clear water fish had an affinity towards the visual stimulus. So differences in behaviour were observed and these could be related to the size of the associated brain region.

Changes to the benefits of relying on different sensory modalities would be expected to dramatically change the direction and targets of selection, altering evolutionary lines within species and increasing the potential for the maintenance and production of new species. This is termed sensory drive. Whilst evidence is scarce, Seehausen *et al.,* (2008) presented a study using island populations of African cichlid fish *Pundamilia spp.* as a model system and demonstrated that female preferences for male mate colouration across species was consistent with ambient light colour within the habitat they reside, with females choosing males of the most conspicuous colouration. If speciation can result

through sensory drive, the results of this study showing that species can adapt their sensory preferences through neuro-morphological and behavioural changes, it wouldn't be unreasonable to predict that over successive generations, climate change induced alterations to marine environments could impose selection on different sensory modalities and distinct new species could arise. It has already been demonstrated by Gonda (2011, 2012, 2013) through examination of F1 populations that changes in brain size between different sticklebacks can be genetically inherited. In short, the implications for this study are that over successive generations adapting to environments such as these which cause a shift in sensory preference for social cues, natural selection may occur in sticklebacks and other fish species to produce distinct, reproductively isolated, species.

The quantitative approach used by Lisney *et al.,* (2007) to estimate the size of brain structures from length, width, and height measures of intact brains was utilised throughout both parts of this thesis. We used estimates from multiple samples with the aim of preventing individual variations in brain size from affecting conclusions. A draw back to using the ellipsoid model for estimating brain size is that it might not account for certain shape changes such as differences in the crescent shape of the optic tectum. Such a model might also be have its limitations if there were significant interspecific variations in shape of various structures. These issues were addressed in this study, however, because comparisons were made intraspecifically where drastic shape changes did not occur.

On a broader spectrum, what we have shown here is that individuals possess the ability to adapt their behaviour and morphology to that what is most appropriate to the environment in which they find themselves. Climate change is affecting the planet as a whole but some places are changing differently to others in terms of temperature, acidity and so on and this may give rise to speciation through the different ways that populations of the same species adapt their modalities of such behaviours as communication, shoaling and mate choice.

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